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ORIGINAL RESEARCH

Sodium leak channel, non-selective contributes to the leak current in human myometrial smooth muscle cells from pregnant women

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ABSTRACT: Uterine contractions are tightly regulated by the electrical activity of myometrial smooth muscle cells (MSMCs). These cells require a depolarizing current to initiate Ca^{2+} influx and induce contraction. Cationic leak channels, which permit a steady flow of cations into a cell, are known to cause membrane depolarization in many tissue types. Previously, a Gd^{3+}-sensitive, Na^{+}-dependent leak current was identified in the rat myometrium, but the presence of such a current in human MSMCs and the specific ion channel conducting this current was unknown. Here, we report the presence of a Na^{+}-dependent leak current in human myometrium and demonstrate that the Na^{+}-leak channel, NALCN, contributes to this current. We performed whole-cell voltage-clamp on fresh and cultured MSMCs from uterine biopsies of term, non-laboring women and isolated the leak currents by using Ca^{2+} and K^{+} channel blockers in the bath solution. Ohmic leak currents were identified in freshly isolated and cultured MSMCs with normalized conductances of 14.6 pS/pF and 10.0 pS/pF, respectively. The myometrial leak current was significantly reduced ($P < 0.01$) by treating cells with 10 μM Gd^{3+} or by superfusing the cells with a Na^{+}-free extracellular solution. Reverse transcriptase PCR and immunoblot analysis of uterine biopsies from term, non-laboring women revealed NALCN messenger RNA and protein expression in the myometrium. Notably, ~90% knockdown of NALCN protein expression with lentivirus-delivered shRNA reduced the Gd^{3+}-sensitive leak current density by 42% ($P < 0.05$). Our results reveal that NALCN, in part, generates the leak current in MSMCs and provide the basis for future research assessing NALCN as a potential molecular target for modulating uterine excitability.

Key words: myometrium / leak current / uterus / pregnancy / contraction

Introduction

Regulation of uterine contractile frequency and intensity is essential for several functions of the female reproductive tract, including implantation of the embryo, transport of sperm for fertilization, and formation of contractile waves associated with the menstrual cycle (Fanchin and Ayoubi, 2009). Most notably, the contractile state of the uterus must be tightly regulated throughout pregnancy; the uterus must remain quiescent during pregnancy to foster growth of the fetus, become activated at term to produce forceful, coordinated, and rhythmic contractions to deliver the infant, and finally involute to return to its pre-pregnancy state.

The myometrial smooth muscle cell (MSMC) action potential underlies control of uterine contractions, with the phasic and regenerative depolarization and repolarization of the MSMC membrane resulting in rhythmic, spontaneous contractions. The depolarizing upstroke is predominantly due to Ca^{2+} influx, which is modulated, in large part, by activation of L-type calcium channels. The roles of Ca^{2+} in mediating the membrane potential and providing a contractile signal for electro-mechanical coupling have been well characterized (Berridge, 2008). Recent evidence has revealed that a large milieu of ion channels underlie the electrical activity of MSMCs (Chan *et al.*, 2014), indicating the complexity of signals that must function coordinately to keep MSMCs in a pro- or anti-contractile state. However, the currents that depolarize the membrane to the extent required for L-type Ca^{2+} channel activation have not been fully elucidated.

An important contributor to cell excitability is the action of a non-selective cationic leak current, defined as a non-gated, or constitutively active, flow of cations across the cell membrane with a reversal potential nearing 0 mV under physiological ion concentrations. For example, in the sinoatrial node of the heart, the inwardly rectifying K^{+} channels and the

hyperpolarization-activated 'funny' current provide a coordinated leak of cations into cardiomyocytes to produce pacemaker activity (DiFrancesco, 1981; Nichols *et al.*, 1996; DiFrancesco, 2010). Similarly, the rhythmic firing of dopaminergic neurons of the ventral tegmental area of the brain is modulated by a tetrodotoxin-insensitive Na^+-leak current (Khaliq and Bean, 2010).

Working with rat MSMCs, Miyoshi *et al.* (2004) observed a leak current that displayed a Na^+-dependent conductance and was sensitive to inhibition by the trivalent cation gadolinium (Gd^{3+}) (IC_{50} of 3.0 μM). No such leak current has yet been identified in the human myometrium. Furthermore, the specific ion channels that drive this current remain to be identified. RNA sequencing data from human myometrial tissue revealed the expression of a channel with leak-distinctive features, the Na^+ leak channel, non-selective (NALCN) (Chan *et al.*, 2014). This channel is a member of the 4 × 6 transmembrane domain family, which includes voltage-gated Ca^{2+} and voltage-gated Na^+ channels (Lee *et al.*, 1999; Yu and Catterall, 2004; Lu *et al.*, 2007). Studies of NALCN in heterologous expression systems, mice, *Caenorhabditis elegans*, *Lymnaea stagnalis* and *Drosophila melanogaster* nervous systems have demonstrated that, unlike other members of its family, NALCN is voltage-insensitive and contains a non-selective pore domain, allowing it to conduct Na^+, Ca^{2+}, K^+ and Cs^+ (Lear *et al.*, 2005; Lu *et al.*, 2007; Yeh *et al.*, 2008; Lu and Feng, 2011; Senatore *et al.*, 2013). In addition to its canonical form, NALCN has several alternatively spliced isoforms with undescribed functions (Senatore *et al.*, 2013; Cochet-Bissuel *et al.*, 2014). NALCN is sensitive to inhibition by Gd^{3+} (IC_{50} of 1.4 μM) (Lu *et al.*, 2007, 2010; Swayne *et al.*, 2009) and provides properties similar to the current described in the rat myometrium (Miyoshi *et al.*, 2004).

By conducting a leak current, NALCN has a profound effect on excitable cells. NALCN mediates the slow influx of Na^+ into the cell, bringing the resting membrane potential closer to the threshold for action potential generation (Lu *et al.*, 2007; Yeh *et al.*, 2008; Lu and Feng, 2011). This characteristic allows it to drive rhythmic processes including respiratory rhythm, gastro-intestinal contractility and circadian behavior (Lear *et al.*, 2005; Lu *et al.*, 2007; Kim *et al.*, 2012), making it a strong candidate for the regulation of uterine activity. On the basis of evidence that NALCN mRNA is expressed in the human myometrium and its role in conducting an excitatory leak current in multiple rhythmic cell types, we hypothesized that the NALCN channel is expressed in the human myometrium and contributes to a cationic leak current in MSMCs. We addressed our hypothesis by using electrophysiology and RNA inhibition technology in freshly isolated and cultured MSMCs from pregnant women at term. We report that human MSMCs have a Na^+-dependent, Gd^{3+}-sensitive leak current that is dependent on NALCN expression. The discovery of NALCN expression and activity in the myometrium has implications for the modulation of uterine contractility and reproductive function in women.

Materials and Methods

Ethical approval and acquisition of human samples

Human myometrial tissue from the lower uterine segment was obtained from 23 non-laboring women who underwent Cesarean hysterotomy under regional anesthesia in late pregnancy (term non-labor [TNL]; 37–40 weeks of gestation). The indications for Cesarean section were placenta previa (1 woman), non-reassuring fetal status (1), breech presentation (1), previous delivery with shoulder dystocia (2), hip replacement (1) and previous Cesarean section (17). None of the women reported having contractions, and no contraction-altering drugs were given. Before the procedure, a clinical research nurse coordinator in the Department of Obstetrics and Gynecology at Washington University School of Medicine explained details of the biopsy procedure to the subjects and obtained signed written consent forms approved by the Washington University in St. Louis Human Research Protection Office (IRB 201108143). The subject population was ~50% African American and 50% Caucasian. Of the 23 women, 9 reported an underlying health problem(s), including mental illness (3 women, 1 of whom was taking medication), hypotension (1), gestational hypertension (1), history of syncopal episode (1), Bell's Palsy (1), morbid obesity (1), rheumatoid arthritis (1) and an ovarian cyst (1). Additionally, one woman's fetus was large for gestational age, and another woman's fetus presented with intrauterine growth restriction. None of the data acquired from samples from women with underlying disease was outside the normal range, and analysis was done blinded to the underlying conditions. Samples were stored in ice-cold phosphate buffered saline (PBS) and processed for explant expansion and smooth muscle cell isolation within 90 min of acquisition. Samples used for RNA extraction were collected in RNA*later*® (Ambion, Austin, TX, USA) and stored at −20°C within 30 min of acquisition. Samples used for membrane preparations were flash frozen in liquid nitrogen and stored at −80°C within 90 min of acquisition. Samples obtained from at least three women were used for each experiment.

Cell isolation

Freshly isolated human myometrial smooth muscle cells (hMSMCs) were isolated from myometrial samples according to previously described methods (Tribe *et al.*, 2000) with minor modifications. Briefly, samples were cut into pieces ~2–4 mm^2 and digested with 1 mg/ml collagenase IA (Sigma, St. Louis, MO, USA) and 1 mg/ml collagenase XI (Sigma) in DMEM F-12 media (Ref. 11039, Gibco, Grand Island, NY, USA) containing 0.1% BSA (Sigma) and 25 μg/ml gentamicin (Gibco) for 40 min at 37°C. Digested tissue was strained through a 40-μm cell strainer, washed twice with DMEM F-12 containing 10% FBS (Ref. 26140, Gibco) and 25 μg/ml gentamicin (standard media), and then plated onto a six-well plate in DMEM F-12 containing 5% FBS and 25 μg/ml gentamicin. Freshly isolated cells were used within 3 and 22 h after tissue biopsy.

Cultured hMSMCs from TNL explants were expanded within 2 h of biopsy in DMEM F-12 medium supplemented with 5% FBS, 2 ng/ml basic fibroblast growth factor (Lonza, Allendale, NJ, USA), 3 ng/ml epithelial growth factor (Lonza), 25 μg/ml gentamicin, 5 μg/ml fungizone (Gibco), and 5 μg/ml insulin (Sigma). Cells were maintained in standard media and used at passage four. Immunoblotting confirmed that NALCN expression was maintained through this passage. Quantitative RT–PCR showed that NALCN expression was higher in passage four than in passage one.

Electrophysiology

Cells were plated directly or trypsinized and transferred to glass coverslips, and electrophysiological measurements were made within 4 h. Cultured hMSMCs were serum starved in 0.5% FBS-containing media for 20–24 h before electrophysiology experiments were performed. Glass pipettes were pulled and polished to 2.5–5 MΩ and filled with a pipette solution containing 125 mM Cs-Aspartate, 20 mM tetraethylammonium (TEA)-Cl, 5 mM Mg-ATP, 5 mM EGTA, 100 nM free Ca^{2+} (calculated with Maxchelator software [Stanford University]), and 10 mM HEPES, pH 7.2. Currents were measured in an extracellular bath solution containing: 125 mM NaCl or *N*-methyl-D-glucamine (NMDG), 20 mM TEA-Cl, 0.1 mM $MgCl_2$, 5 mM HEPES, 11 mM glucose, 1 μM $CaCl_2$, and 5 μM nifedipine, pH 7.4. K^+ was

replaced with Cs^+. TEA-Cl and nifedipine were added to the bath solution to isolate the leak current by blocking voltage-gated K^+ and Ca^{2+} channels, respectively. Leak currents were recorded at a sampling rate of 10 kHz and filtered at 1 kHz by using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Cells were held at 0 mV and currents were elicited by stepping from −55 mV (50 ms) to +40 mV for 100 ms (to inactivate voltage-gated Ca^{2+} and K^+ channels) followed by 20 mV steps (500 ms) from −100 mV to +100 mV using the pClamp 10 software (Molecular Devices). Currents were normalized to cell capacitance, and the mean and standard error of the mean (SEM) of the current density were calculated for each group of samples. Normalized conductance was determined by calculating the slope of the linear regression for currents elicited by voltages between −100 mV and −20 mV, as this was the most linear part of the currents. Reversal potentials were similarly determined by calculating the X-intercept of the linear regressions and calculating the mean ± SEM.

RNA isolation and RT–PCR

Total RNA was isolated from myometrial biopsies by using the Aurum Total RNA Fatty and Fibrous Tissue Pack Kit (BioRad, Hercules, CA, USA) and reverse transcribed to generate cDNA by using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. cDNA was stored at −20°C until PCR was performed. Forty-cycle PCR was performed on cDNA by using Taq PCR Master Mix (2X) (Affymetrix, Santa Clara, CA, USA) and primers (Table I) specific to NALCN, UNC80, UNC79, or NLF-1 (Integrated DNA Technologies, Coralville, IA, USA). Products were electrophoresed on a 1.5% agarose gel.

RNAi lentiviral transduction

Replication-deficient lentiviruses were packaged by the England lab or by the HOPE Center Viral Vectors Core at Washington University in St. Louis. The lentiviral vector, pLVTHM, was a gift from Arnaud Monteil (Swayne *et al.*, 2009). The encoded short hairpain (sh) RNA target sequences were designed by an Ambion algorithm to be specific to NALCN and have no more than 70% coverage with any other part of the human transcriptome. The target sequences are reported in Table I. hMSMCs were incubated with half the normal flask volume (6 ml for a T-75, 1 ml for a 6-well plate) of standard media containing 8 μg/ml polybrene for 10–15 min before addition of the viral particles. Transductions took place over 16 h, after which media was replaced with standard media. Only cells expressing GFP (co-expressed by the viral vector and confirmed by fluorescence microscopy) were used for electrophysiological measurements. For analysis of knockdown, an MOI (multiplicity of infection) that generated 85–95% GFP-positive cells (confirmed by flow cytometry 48 h after transduction) was used. Cells were used 6–8 days post-transduction for electrophysiology and 6–20 days post-transduction for qRT–PCR and western blot analyses.

Table I DNA primer and shRNA target sequences.

Primer name	Sequence
NALCN_F	ACCATGGGTTCACTCTTTGC
NALCN_R	TGCTATCATCTCTGCCGTGT
UNC80_F	TCAGTCTGGTCAGTGCGTTC
UNC80_R	ACCTCACCATCCTCCATCAG
UNC79_F	TGGACCACAATTAGCAACCA
UNC79_R	CCAGGTAGGCCAGAGTGAAG
NLF-1a_F	CACAGGACATCGCTCACAGT
NLF-1a_R	TTTCTTCCAGCGTGTTGAT
SDHA_F	CAAACAGGAACCCGAGGTTTT
SDHA_R	ATACAGCATGTGTTACCAAGCTG
TOP1_F	CCAGACGGAAGCTCGGAAAC
TOP1_R	TTCAAGATAGAGCCTCCTGGAC
shRNA name	**Target sequence**
shNALCN*	AATGTATGACATAACCCAGCA
shScrambled*	GCTCAGTACGATCATACTCAC

*Previously published in Swayne *et al.* (2009).

Quantitative RT–PCR

RNA was extracted from hMSMCs 6–9 days after transduction with lentiviral vectors. RNA quality was ensured by gel electrophoresis analysis and 260/280 nm and 260/230 nm absorbance ratios above 1.8 and 2.0, respectively. qRT–PCR reactions (20 μl) contained 5 pmol of each forward and reverse primer (Table I), cDNA produced from 50 ng of RNA, and iQ SYBR Green Supermix (BioRad). Gene targets were amplified and quantified by the CFX96 BioRad Real-Time PCR Detection System. The temperature cycles were as follows: 95°C for 3 min (1×), and 95°C for 10 s followed by 57°C for 30 s (40×), ending with a 0.5°C increment melt curve from 65 to 95°C. Primer efficiencies were ≥77% with human brain cDNA, and the calculated efficiency values were used in Pfaffl analysis of relative gene expression (Pfaffl, 2001). Target gene expression was normalized to the reference genes topoisomerase I (TOP1) and succinate dehydrogenase complex, subunit A (SDHA), both chosen because of their consistency of expression in the myometrium across pregnancy (Rosenbaum *et al.*, 2012).

Western blot analysis

For lysis of cultured hMSMCs, media was aspirated from the cells, and they were washed with cold DPBS. Cells were then scraped into ice-cold lysis buffer containing: 10 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, and a Complete Mini Protease Inhibitor Cocktail pellet (EDTA-free, Roche Molecular Biochemicals, Indianapolis, IN, USA), pH 8.0. Cells were then homogenized with 0.1-mm glass beads by using the Next Advance Bullet Blender (Averill Park, NY, USA). Homogenates were separated by centrifugation at 9000*g* for 20 min at 4°C.

For preparation of human myometrium cell membranes, flash frozen tissue was finely chopped and then homogenized by a Tekmar Tissumizer (Cincinnati, OH) in membrane preparation solution containing: 250 mM sucrose, 50 mM MOPS, 2 mM EDTA, 2 mM EGTA, pH7.4, 1 mM PMSF, and a Complete Mini Protease Inhibitor Cocktail pellet (EDTA-free). Non-homogenized tissue was removed by centrifugation at 14 000*g* for 15 min. Cell membranes were isolated from the supernatant by ultracentrifugation at 100 000*g* for 1 h. Membrane pellets were dissolved in lysis buffer for 2 h. Any remaining insoluble components were removed by centrifugation at 9000*g* for 20 min. All steps were performed at 4°C with ice-cold buffers to prevent proteins from degrading.

Protein concentrations of whole cell lysates and membrane preparations were measured by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Protein samples were mixed with Laemmli sample buffer, heated to 65°C for 15 min, and stored at −20°C. Protein samples (40 μg for myometrium, and 10 μg for mouse brain) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking in PBS containing 0.075% Tween-20 (PBST) and 5% milk, membranes were probed with the following primary antibodies: anti-NALCN N-20 (1:500; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA, USA), anti-GAPDH (1:1000; Millipore, Billerica, MA, USA), or anti-transferrin receptor (1:500; Invitrogen, Camarillo, CA, USA). Blots were then probed with HRP-conjugated secondary antibodies (1:4000; Jackson ImmunoResearch Laboratory Inc., West Grove, PA, USA) in PBST containing 3% milk. Signal was detected with Clarity ECL Western chemiluminescence

(BioRad). NALCN expression was normalized to GAPDH by using ImageJ densitometry, and values from shNALCN-treated cells were compared with those from shScrambled (Scr)-treated cells.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was determined by one-tailed Student's *t*-test (two groups), or one- or two-way ANOVA (three or more groups) correcting for multiple comparisons by using GraphPad Prism 6 (San Diego, CA, USA), and $P < 0.05$ was considered significant. *N* refers to number of cells, and *n* refers to the number of patient samples tested.

Results

Human MSMCs conduct a Na^+-dependent, Gd^{3+}-sensitive leak current

To determine whether a leak current is expressed in the human myometrium, whole-cell voltage clamp experiments were performed on freshly isolated human myometrial smooth muscle cells (hMSMCs) from uterine samples from non-laboring women who had Cesarean sections at full term. An ohmic leak current with a reversal potential of 21.8 ± 9.2 mV was identified (Fig. 1A and C and Table II). Addition of 10 μM

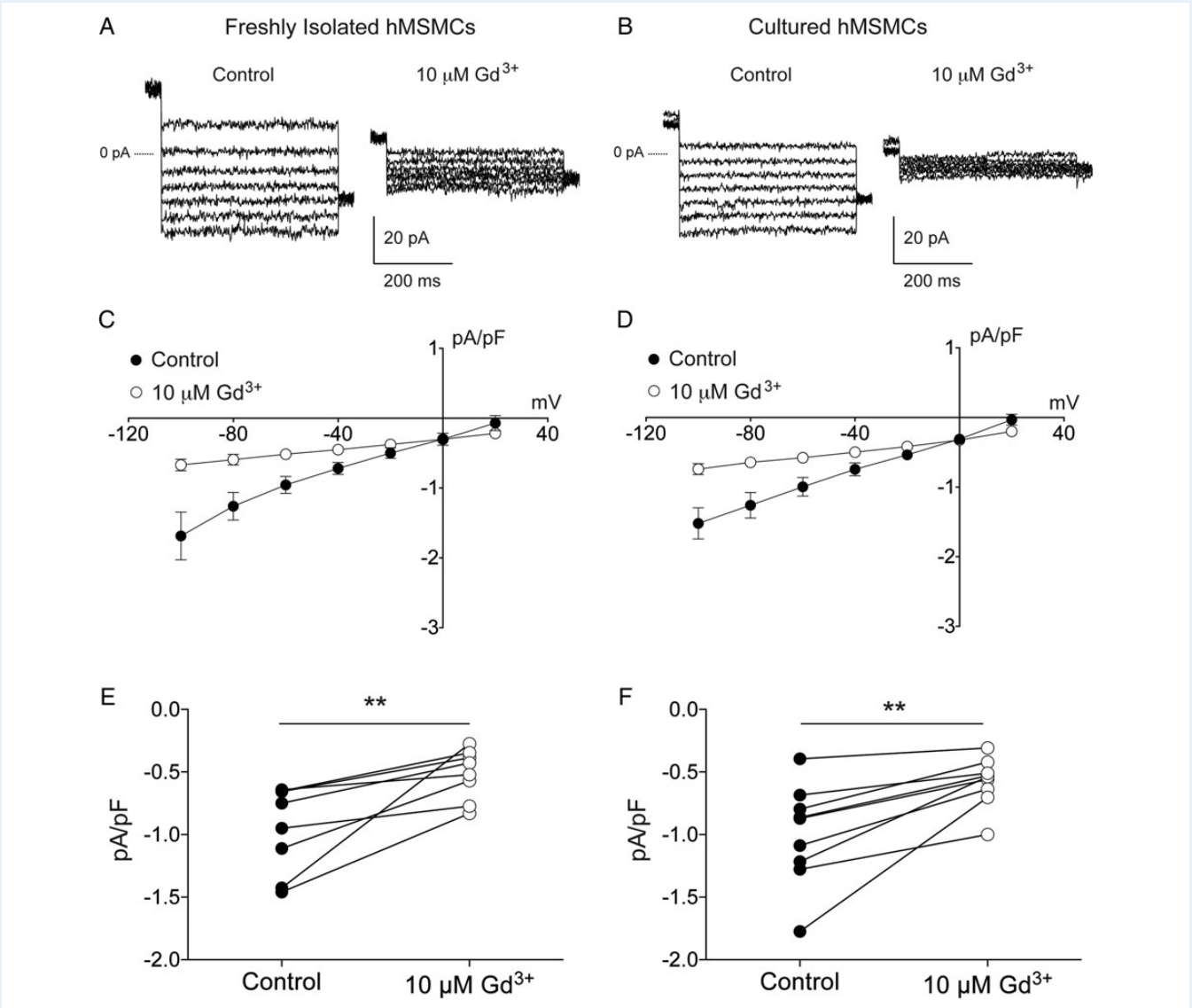

Figure 1 Freshly isolated and cultured hMSMCs conduct a Gd^{3+}-sensitive leak current. (**A** and **B**) Representative traces of leak current evoked from freshly isolated hMSMC (A) and cultured hMSMC (B) by using a voltage step protocol with 20 mV increments from −100 mV to +20 mV, before and after treatment with 10 μM Gd^{3+}. Capacitive currents were removed from these traces. (**C** and **D**) Current-voltage relationships obtained from freshly isolated (C, $N = 7$) and cultured (D, $N = 7$) hMSMCs before (closed circles) and after (open circles) treatment with 10 μM Gd^{3+}. Data are presented as mean values ± SEM. (**E** and **F**) Current density analysis from freshly isolated (E) and cultured (F) hMSMCs at −60 mV before and after treatment with 10 μM Gd^{3+}. Symbols represent individual cells, and connecting lines link values in the same cell sample before and after treatment with Gd^{3+}. $^{**}P = 0.0039$ (E), and $P = 0.0020$ (F).

Table II Summary of leak current parameters in hMSMCs.

Treatment (*n*)	Calculated reversal potential (mV)	Normalized conductance (pS/pF)	Current density[#] (pA/pF)
Freshly isolated cells (11)	21.8 ± 9.2	14.6 ± 4.0	−0.96 ± 0.12
+10 μM Gd^{3+}	88.0 ± 17.1**	3.6 ± 0.5**	−0.52 ± 0.07**
Cultured Cells (18)	31.7 ± 4.3	10.0 ± 1.3	−0.84 ± 0.08
+10 μM Gd^{3+} (9)	86.1 ± 6.5**	4.0 ± 0.5**	−0.56 ± 0.04**
NMDG (9)	66.6 ± 12.9**	3.5 ± 0.4**	−0.42 ± 0.04**
shScr (11)	16.8 ± 5.1	9.4 ± 1.6	−0.64 ± 0.07
+10 μM Gd^{3+}	57.7 ± 11.8[b]	2.7 ± 0.5[b]	−0.28 ± 0.03[b]
shNALCN (9)	28.9 ± 5.2	5.9 ± 0.8[a]	−0.51 ± 0.04
+10 μM Gd^{3+}	75.6 ± 13.0[b]	2.6 ± 0.5[b]	−0.30 ± 0.03[b]

Mean values ± SEM.
[#]Calculated at −60 mV.
*$P < 0.05$ and **$P < 0.01$ from paired Student's *t*-test comparing before and after treatment with Gd^{3+} or NMDG-containing solution.
[a]$P < 0.05$ from multiple-comparisons two-way ANOVA between shScr and shNALCN groups.
[b]$P < 0.01$ from matched, multiple-comparisons two-way ANOVA between before and after treatment with Gd^{3+}.

Gd^{3+} (a concentration known to inhibit NALCN currents by ~80%) (Lu *et al.*, 2007) to the bath solution significantly reduced the average conductance of this current by 75% and shifted the reversal potential to 88 mV (Table II), indicating the presence of a Gd^{3+}-sensitive current in freshly isolated cells. Although freshly isolated hMSMCs are optimal for characterizing myometrial cell biology, they cannot be used for repeated and longer-term experiments. The same whole-cell voltage clamp experiments revealed that, qualitatively, cultured hMSMCs behaved like freshly isolated hMSMCs (Fig. 1B and D). As in freshly isolated hMSMCs, currents elicited in cultured cells at −60 mV, near the resting membrane potential, were significantly inhibited by addition of Gd^{3+} (Fig. 1E and F).

To elucidate the predominant cation contributing to the current in cultured hMSMCs, Na^+ in the bath solution was replaced with the channel-impermeable monovalent cation, NMDG. This reduced the conductance (Fig. 2A, B and Table II) to a similar extent as the addition of Gd^{3+} (Fig. 1D). As anticipated, the reversal potential with NMDG was more similar to the control (unlike the large positive shift seen with Gd^{3+} treatment [Fig. 1D]), indicating that Na^+ replacement significantly inhibited the inward portion of the leak current (Fig. 2A). To determine the extent to which the leak current was active at a physiologically relevant membrane potential, currents were recorded while holding cultured hMSMCs at −60 mV (Fig. 2C) (Parkington *et al.*, 1999). Although Na^+-dependent current density varied noticeably between individual hMSMCs, replacement with NMDG resulted in a significant reduction of the average current density elicited at −60 mV (Fig. 2B and Table II). Finally, to determine the extent to which Na^+ drives the Gd^{3+}-sensitive current, 10 μM Gd^{3+} was added to the NMDG superperfusate, and any further reduction in the current density was analyzed (Fig. 2B). Gd^{3+} did not significantly inhibit the leak current any further, indicating that Na^+ is the major cation driving the Gd^{3+}-sensitive inward current. Overall, these data suggest that the Gd^{3+}-sensitive current in cultured hMSMCs is largely driven by Na^+ conductance.

NALCN activity contributes to the myometrial leak current

Given that NALCN conducts a Na^+-dependent, Gd^{3+}-sensitive leak current and, like the myometrial leak current, can be modulated by G-protein coupled receptor (GPCR)-mediated signaling (Marshall, 1959; Lodge and Sproat, 1981; Lu *et al.*, 2009, 2010; Swayne *et al.*, 2009), NALCN was a prime candidate for the specific ion channel that conducts the myometrial leak current. To assess this possibility, NALCN expression was assessed in the human myometrium. NALCN mRNA expression was detected in uterine tissue from pregnant term non-laboring (TNL) women by using non-quantitative RT−PCR (Fig. 3A). Protein expression of NALCN was confirmed in TNL myometrial tissue by immunoblot (Fig. 3B). In hippocampal and ventral tegmental area neurons in the mouse and premotor interneurons in nematodes, modulation of NALCN by GPCRs requires the presence of two putative scaffolding proteins, UNC80 and UNC79, and a localization factor (NLF-1) (Yeh *et al.*, 2008; Lu *et al.*, 2009, 2010; Xie *et al.*, 2013) (Fig. 3B). Non-quantitative RT−PCR showed that whereas UNC79 and NLF-1a were expressed in TNL human myometrial tissue, UNC80 was not (Fig. 3A). However, UNC80 expression was detected in uterine tissue from non-pregnant women (data not shown).

Importantly, Gd^{3+} has many ion channel targets (Yang and Sachs, 1989; Lacampagne *et al.*, 1994) and, to date, no specific blockers of NALCN exist. Thus, to assess the contribution of NALCN to the leak current in hMSMCs, shRNAs were used to knock down its expression. Cultured hMSMCs were transduced with lentiviral vectors expressing shRNAs that target all isoforms of NALCN. NALCN mRNA was decreased by 50%, and protein expression was reduced by 80−90% (Fig. 4A and B); the level of transcript knockdown matches that previously observed by Swayne *et al.* (2009). NALCN levels did not notably differ between cells treated with transduction additive, polybrene, alone and those transduced with shScr (Fig. 4B). Cells in which NALCN expression was reduced with shRNAs had 37% lower average leak current conductance and 20% lower average leak current density at −60 mV than control cells (Fig. 4C and Table II). However, knockdown by shNALCN did not reduce the current to the same extent as 10 μM Gd^{3+}. Importantly, Gd^{3+} does not completely abolish the leak current, likely because Gd^{3+}-insensitive channels contribute to the current or inherent leak exists in the patch clamp seal. Therefore, the contribution of NALCN to the Gd^{3+}-sensitive leak current alone can be calculated by subtracting the Gd^{3+}-insensitive current. This revealed that NALCN knockdown reduced the Gd^{3+}-sensitive current density at −60 mV by an average of 42%

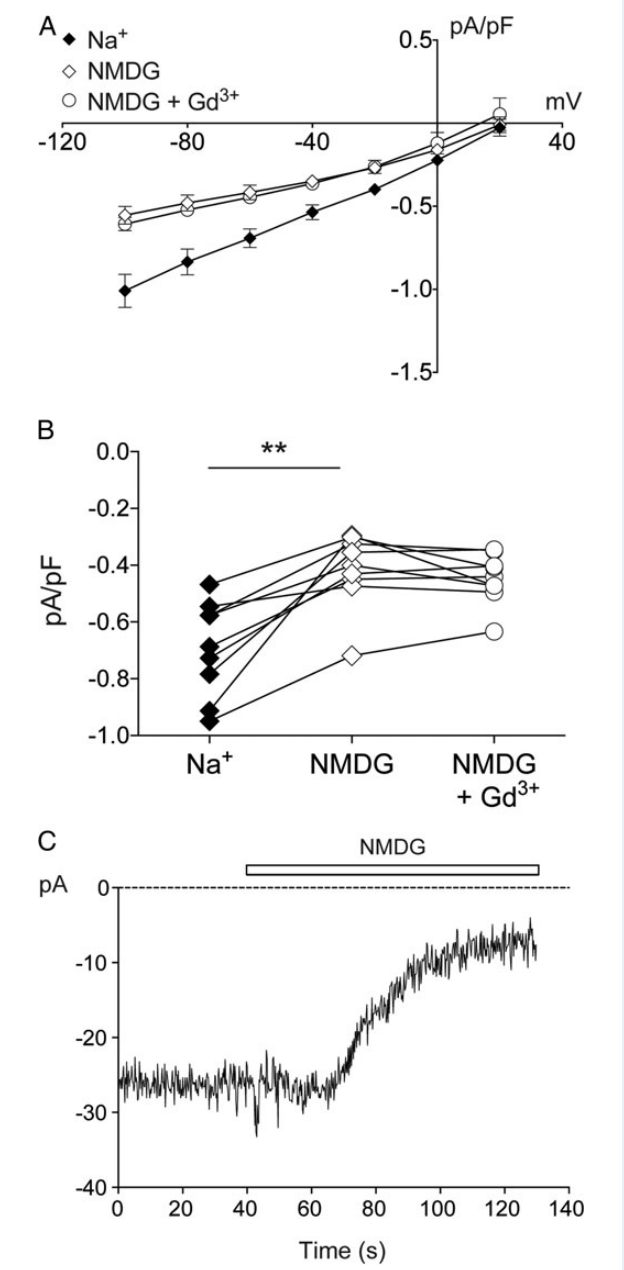

Figure 2 Cultured hMSMCs conduct a Na^+-dependent leak current. (**A**) Current-voltage relationship observed in cultured hMSMCs superfused with bath solution containing 125 mM Na^+ (closed diamonds) and replaced with 125 mM NMDG (open diamonds) or 125 mM NMDG + 10 μM Gd^{3+} (open circles) ($N = 9$). Data are presented as mean values ± SEM. (**B**) Current density analysis at −60 mV from voltage step protocol in (A) before (Na^+, closed diamonds) and after perfusion with NMDG (open diamonds) and NMDG + Gd^{3+} (open circles) ($N = 9$). Symbols represent individual cells, and connecting lines link values before and after each treatment. $^{**}P = 0.0020$. (**C**) Representative current from a cultured hMSMC clamped at −60 mV. Na^+-containing bath solution was replaced with NMDG-containing solution at the indicated time.

(Fig. 4D). Taken together, these data are consistent with the notion that NALCN significantly contributes to the Na^+-dependent, Gd^{3+}-sensitive leak current in cultured hMSMCs.

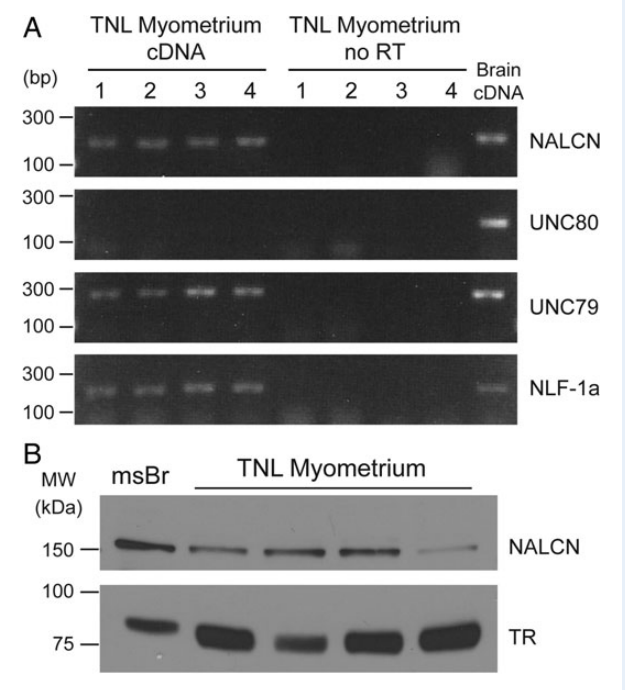

Figure 3 mRNA and protein expression of NALCN in human myometrium. (**A**) Representative RT–PCR of NALCN complex subunits (UNC79, UNC80 and NLF-1a) from human myometrial samples from four pregnant term non-labor (TNL) women. Negative (no reverse transcriptase [RT]) and positive (human brain cDNA) controls are also shown. (**B**) Representative immunoblot of NALCN from TNL human myometrial sample membrane preparations from four different women. A mouse brain (msBr) membrane preparation served as a positive control. Transferrin receptor (TR) served as a loading control.

Discussion

Uterine contractions are regulated by the coordination of depolarizing and repolarizing currents mediated by ion channel activity. Here, we present the identification of a likely source of myometrial excitation: a cationic leak current. This current was Gd^{3+}-sensitive, Na^+-dependent, and present in both freshly isolated and cultured hMSMCs. This is the first demonstration that NALCN is expressed in the human myometrium and that its expression significantly contributes to the leak current. Notably, knockdown of NALCN expression significantly reduced the leak current at a physiologically relevant membrane potential of −60 mV.

We found that NALCN-targeting shRNAs were able to reduce the Gd^{3+}-sensitive conductance by 42% in hMSMCs. This substantial reduction indicates a significant contribution by NALCN to the leak current. Nonetheless, NALCN knockdown did not abolish the leak current in hMSMCs. Although it is possible that the remaining ~10–20% of NALCN channels were capable of carrying 58% of the cell's leak current, it is more likely that the remaining Gd^{3+}-sensitive (and also Na^+-dependent) current is driven by additional channels that contribute to the leak current in MSMCs. For example, several transient receptor potential channels, which are sensitive to inhibition by Gd^{3+}, have been described to contribute to MSMC currents (Dalrymple *et al.*, 2002, 2007; Shlykov *et al.*, 2003; Murtazina *et al.*, 2011). Additionally, Gd^{3+} and NMDG have recently been shown to block non-specific

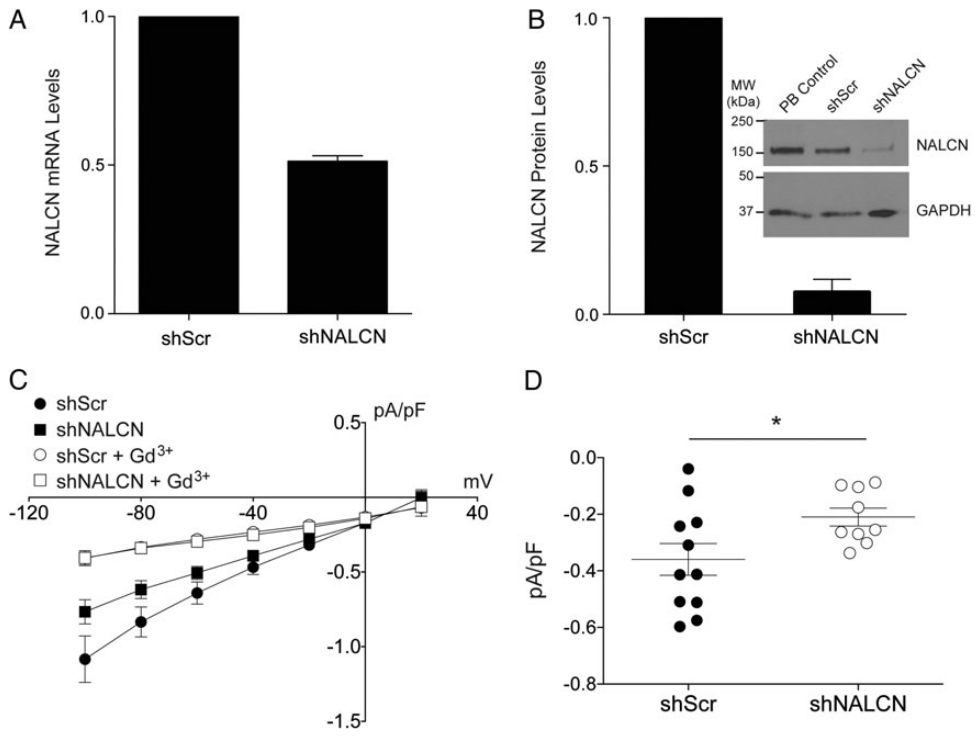

Figure 4 NALCN contributes to the Gd^{3+}-sensitive leak current in hMSMCs. (**A**) qRT–PCR analysis of NALCN mRNA levels after transduction with shRNAs. Bars represent the mean values ± SEM. Levels were normalized to SDHA and TOP1 and compared with scrambled shRNA (shScr) levels ($n = 3$). (**B**) Quantification of NALCN protein expression from immunoblots of cultured hMSMCs treated with polybrene alone or transduced with lentiviruses expressing shScr or NALCN-targeting shRNA (shNALCN). Bars represent the mean values ± SEM, normalized to shScr levels ($n = 4$). Inset shows a representative immunoblot. GAPDH served as loading control. (**C**) Current-voltage relationships obtained from cultured hMSMCs transduced with shRNAs before and after treatment with 10 μM Gd^{3+}. Symbols represent mean values ± SEM ($N = 9–11$). (**D**) Gd^{3+}-sensitive current density analysis at −60 mV. Symbols represent individual cells. Lines represent mean values ± SEM. *$P = 0.0401$.

leak currents in whole-cell recordings with poor seal quality of less than 1 GΩ (Boone *et al.*, 2014). However, we assured membrane seals of ≥1 GΩ in our experiments and therefore conclude that the inhibition observed by treatment with Gd^{3+} and removal of Na^+ from the bath solution is channel specific.

The low level of NALCN activity observed in hMSMCs could be due to the fact that the uterine tissue we used was biopsied from the lower uterine segment. This segment of the uterus has been shown to be less contractile than the uterine fundus (Fuchs, 1969; Crane and Martin, 1991) and may express fewer excitatory ion channels such as NALCN. Furthermore, we did not detect mRNA expression in TNL myometrium of an important NALCN complex subunit, UNC80. This finding is consistent with an observation that HEK293 cells have NALCN leak activity in the absence of UNC80 co-expression (Lu *et al.*, 2007). Therefore, although GPCR-mediated modulation of NALCN activity requires UNC80 expression, baseline NALCN leak activity appears to function independent of canonical UNC80. Controversy exists in the field as to whether NALCN is a true leak channel or is an agonist-induced (through GPCRs) non-selective, voltage-insensitive channel. Although our work indicates that NALCN functions as a leak channel in MSMCs, MSMCs may express low-level constitutive GPCR activity that keeps NALCN active in these cells, as discussed by Swayne *et al.* (2009). Acetylcholine and substance P, two agonists that increase uterine contractility, have been shown to activate NALCN in neurons, pancreatic β cells, and interstitial cells of Cajal (Marshall, 1959; Lodge and Sproat, 1981; Kitazawa *et al.*, 1999, 2008; Patak *et al.*, 2000; Lu *et al.*, 2009; Swayne *et al.*, 2009; Kim *et al.*, 2012). Thus, the involvement of NALCN in agonist-induced contractility is an important subject for future research.

Establishing the source of a non-selective leak current in the human myometrium fills an important knowledge gap in the field of myometrial physiology. Clues to the existence of this current have been reported over the last several decades. Miyoshi *et al.* described a Na^+-dependent Gd^{3+}-sensitive leak current in the rat myometrium in 2003, but the channel conducting this current had not yet been identified. In 1971, Anderson *et al.* described a transient Na^+- and Ca^{2+}-conducting current in the rat myometrium that they hypothesized to be due to a single channel (Anderson *et al.*, 1971). They found that a low concentration of extracellular Ca^{2+} enhanced the leak current and that the membrane potential was reduced by nearly 10 mV in a Na^+-free solution. NALCN is a good candidate to generate this current as this channel is activated by low extracellular Ca^{2+} through its regulation by the Ca^{2+}-sensing receptor (Lu *et al.*, 2010) and drives Na^+-dependent membrane depolarization (Lu *et al.*, 2007). Studies also suggest that uterotonins such as acetylcholine increase the rate of membrane depolarization and increase the frequency of action potentials in the myometrium (Marshall, 1959;

Lodge and Sproat, 1981). Given that NALCN can be activated by acetylcholine through muscarinic receptor 3 in pancreatic beta cells (Swayne *et al.*, 2009), NALCN may play roles not only in spontaneous MSMC activity, but also in agonist-induced myometrial excitability.

Through its effect on resting membrane potential, NALCN has been linked to the regulation of rhythmic activity and overall membrane excitability in a variety of biological systems (Lear *et al.*, 2005; Lu *et al.*, 2007; Yeh *et al.*, 2008; Lu and Feng, 2011; Kim *et al.*, 2012). The discovery of its activity in the myometrium could have implications for all stages of reproduction, including sperm transport, embryo implantation, embryonic gestation and parturition. These processes require a range of activity, including complete quiescence, subtle directional peristalsis, and rhythmic, maximal force contractions of the uterus. Considering NALCN's effect on excitability, it is reasonable to hypothesize that altering NALCN expression or activity could significantly alter the contractile state of the uterus. In fact, the resting membrane potential of the human uterus changes throughout gestation (Parkington *et al.*, 1999) and likely drives the unique contraction phenotype of each phase. Thus, an important next step will be to measure the contribution of NALCN to the resting membrane potential and overall uterine excitability.

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Authors' roles

E.L.R.: study design, execution, analysis, manuscript drafting and critical discussion. R.C.: study design and critical discussion. I.A.G.: execution of the study. A.G.C.: study design and critical discussion. S.K.E.: study design, manuscript drafting and critical discussion.

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Conflict of interest

None declared.

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